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RESEARCH ARTICLE

COMPARATIVE ANALYSIS OF TECHNIQUES FOR SEX DETERMINATION THROUGH DENTAL PULP BARR-BODIES: FORENSIC STUDY

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ABSTRACT

Introduction: It is an established fact that sex of an individual can be determined from the dental pulp tissue in living as well as the dead using Barr-bodies. However to establish that which technique is better suited for Barr-body estimation is yet to be compared.
Aim: The objective of the study is to compare histopathology and cytopathology techniques for determination of Barr-bodies in human dental pulp tissue.
Material & Method: A study sample of 40 teeth all extracted for the purpose of orthodontic or prosthodontic treatment and periodontically compromised teeth were selected. The samples were divided into 4 groups of 10 teeth each, which were sectioned using a carborundum disc for obtaining the pulp tissue in toto. The group 1 and 2 (group1- 10 male samples, group2- 10 female samples) were processed conventionally to prepare tissue sections and were used for histopathological analysis. Tissues from group 3 and 4 (group3- 10 male samples, group4- 10 female samples) were crushed and centrifuged at 1000rpm for 10 minutes. The supernatant was then used to prepare the smear and were used for cytopathological analysis. The slides thus obtained were stained with conventional Haematoxylin and Eosin stain in order to locate and identify Barr-bodies.
Statistical analysis: The mean Barr-body count was more in females than in male samples and in histopathological analysis as compared to cytopathological technique. Also cell morphology was better appreciated in histopathology than in cytopathology.
Results & Conclusion: The cytopathology technique is effective while looking for these structures in commonly studied buccal mucosal smears. Though the histopathology technique is more time consuming but gives an excellent result in Barr-body identification through dental pulp tissue. Our study is in contraindication to the previous cytological studies reported in the literature.

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INTRODUCTION

Identification of an individual has always been a mainstay of society and civilizations. The identity of a person is one of the important aspects of forensic odontology and science (Veeraraghavan *et al*., 2010). Antemortem and postmortem identification is carried out using various investigatory methods such as anthropology, radiographs, tooth mark analysis, rugoscopy, tooth prints, dental DNA analysis, buccal mucosal smears, oral smears and dental pulp tissue (Pramod *et al*., 2012). Dental pulp tissue acts as a good source for DNA analysis in deficiency of other sources, as very often the available skeleton tissue and soft tissue specimens are not in appropriate condition. The tooth remains intact and can resist various natural and manmade disasters thus, preserving the soft tissue within (Galdames *et al*., 2010). One of the methods for sex determination is by analyzing the dental pulp Barr-bodies. A Barr-body is X-chromatin in its inactive form that appears as an intranuclear structure or as a mass usually lying against the nuclear membrane in all female somatic cells and is seen only during the Interphase phase of cell cycle. They were first discovered by Barr and Bertam in 1949 (Das *et al*., 2004). They are seen in females only and hence they are often termed as 'CHROMATIN POSITIVE', while the males are deemed 'CHROMATIN NEGATIVE' (Gordon, 1966). In this study histopathological and cytopathological techniques have been employed to determine the presence of Barr-bodies in cells. Histopathology refers to the microscopic examination of a

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surgical specimen or a biopsy by a pathologist, after specimen processing and histopathological sectioning have been done and slides have been prepared. Cytopathology is that branch of science, which deals with the study of cells in terms of structure, function and chemistry. The aim of this study was to compare histopathological and cytopathological techniques for determination of Barr-bodies in human dental pulp tissue. This study was carried out to compare both the above-mentioned techniques for determination of Barr-bodies in dental pulp. The objectives of the study were to compare histopathological and cytopathological techniques for Barr-body identification and to compare differences in gender identification between using both the techniques.

MATERIALS AND METHODS

A total of 40 cases comprising of 20 males and 20 females were studied. Inclusion criteria for the selection of teeth in this study included non-carious teeth, extracted for purpose of orthodontic or prosthodontic treatment and periodontically compromised teeth. Teeth obtained were preserved in 10% Formalin for 24-48 hours at room temperature (25°C) and were divided into 4 groups comprising of 10 teeth each. Group 1 and 2 comprised of 10 Males and 10 Females, respectively and Group 3and 4 of 10 Males and 10 Females, respectively. All the tooth samples were then sectioned using a carborundum disc and the pulp tissue was obtained in toto. For groups 1 and 2: conventional tissue processing techniques and tissue section preparation was done. For groups 3 and 4: cytopathology techniques for smear preparation were carried out.

Technique employed for histopathology

The tooth samples were treated with increasing grades (80%, 90% & 100%) of isopropyl alcohol for 10 minutes each. The dehydrated samples were then transferred to acetone for the next 10 minutes for further dehydration and in xylene, two changes, for 15 minutes each for clearing. The samples were then kept in liquid paraffin for 1 hour for impregnation. The processed tissues were embedded in wax and sections cut with semi-automatic microtome of 3μm thickness and stained with H&E stain.

Technique employed for Cytopathology

Pulp tissues were crushed using mortar and pestle and were then transferred into dry and clean centrifuge tubes containing 5ml solution of 4ml Isopropyl alcohol and 1ml glacial acetic acid for 1 hour fixation in 20 test tubes labelled for each gender. The treated samples in alcohol were then centrifuged for 10 minutes at 1000rpm and the supernatant were collected. The supernatant were again centrifuged for 10 minutes at 1000rpm and the pellets were collected. Cytological smears (10 male and 10 female) were prepared using pellets, air-dried and fixed with few drops of isopropyl alcohol (95%). The smears were then stained with H&E stain. All the stained slides obtained by both the techniques were the viewed under Oil immersion (100x) lens of light microscope (Nikon Eclipse 80i) to study the presence of Barr-bodies.

Procedure employed for H & E staining

The procedure for staining used was progressive hydration in descending grades of alcohol (100%, 90%, 70%) followed by running water rinse. The sections or smears were then stained with Harris's Haematoxylin (NICE, India) for 10 minutes. Sections or smears were washed again in running water and counterstained with Eosin (NICE, India). The sections or smears were then dehydrated with ascending grades of alcohol (70%, 90%, 100%). The final step included clearing in Xylene, mounting with DPX and application of cover slips.

Characterization of Barr-bodies

Barr-bodies appear as clumps of chromatin on the inner nuclear membrane in Interphase nuclei of all somatic cells in females. They are darkly stained and adherent to the inner nuclear membrane. These should not be confused with nucleolus as a Barr-body is seen only in Interphase nuclei of female cells and the nucleolus is located within the cell nucleus as dense chromatin without any definite boundary present in Telophase and the entire Interphase, not adherent to nuclear membrane (Lam *et al*., 2015; Anoop *et al*., 2004).

OBSERVATIONS AND RESULTS

The study was carried out to compare the Histopathology and Cytopathology techniques in Barr-body determination from human dental pulp tissue using following variables-

a) Distinct ability to appreciate Barr-bodies.
b) Cellular and nuclear morphology.
c) Equipment availability.
d) Cost effectiveness.
e) Technique duration.

Four observers independently viewed the slides for inter- and intra-observer variability and following results were derived: The mean Barr-body count value for females came out to be 4.900 ± 0.737 and 1.000 ± 0.816 in histopathological and cytopathological techniques, respectively. For males, it was 2.600 ± 0.843 and 0.600 ± 0.699 in histopathological and cytopathological techniques, respectively. The mean number of Barr-bodies appreciated in case of females (4.9,1.0) was more than that observed in male samples (2.6,0.6) and that in histopathology (4.9,2.6) was more as compared to cytopathological technique (1.0,0.6) (Table 1).

Highly significant values were obtained (P=0.0), on comparing the following categories-

a) Histopathologically & cytopathologically assessed females
b) Histopathologically assessed females & males
c) Histopathologically assessed females & cytopathologically assessed males
d) Histopathologically assessed males & cytopathologically assessed females
e) Histopathologically & cytopathologically assessed males

No significant difference was seen between cytopathologically assessed female and male samples (p value = 0.660) (Table 2). Higher cell numbers were available for study in histopathological technique than cytopathological technique and the cells were better appreciated in former than in later.

Table 1. Barr-Body Count

	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
HF	10	4.9000	0.73786	0.23333	4.00	6.00
CF	10	1.0000	0.81650	0.25820	0.00	2.00
HM	10	2.6000	0.84327	0.26667	1.00	4.00
CM	10	0.6000	0.69921	0.22111	0.00	2.00
Total	40	2.2750	1.86723	0.29523	0.00	6.00

(HF= Histopathologicallyassessed female, CF= Cytopathologically assessed female, HM= Histopathologically assessed male & CM= Cytopathologically assessed male).

Table 2. Post Hoc Test for Multiple Comparisons

Test Appllied	GROUP	Multiple comparison	Mean Difference (I-J)	Std. Error	Sig.
Turkey HSD	HF	CF	3.90000	0.34721	0.000
		HM	2.30000	0.34721	0.000
		CM	4.30000	0.34721	0.000
	CF	HF	-3.90000	0.34721	0.000
		HM	-1.60000	0.34721	0.000
		CM	0.40000	0.34721	0.000
	HM	HF	-2.30000	0.34721	0.000
		CF	1.60000	0.34721	0.000
		CM	2.00000	0.34721	0.000
	CM	HF	-4.30000	0.34721	0.000
		CF	-0.40000	0.34721	0.000
		HM	-2.00000	0.34721	0.000

(HF= Histopathologically assessed female, CF= Cytopathologically assessed female, HM= Histopathologically assessed male & CM= Cytopathologically assessed male)

Table 3. Observer Scores for Cellular and Nuclear Features

C/N (O) Observer	Cell availability (No. of cells)	Cell membrane	Nuclear membrane	X-Chromatin
O1	H-3	H-2	H-3	H-3
	C-1	C-2	C-2	C-2
O2	H-3	H-2	H-3	H-3
	C-1	C-2	C-2	C-2
O3	H-3	H-2	H-3	H-3
	C-1	C-2	C-2	C-2
O4	H-3	H-2	H-3	H-3
	C-1	C-2	C-2	C-2

SCORE: 0- Indistinct, 1- Poorly appreciated, 2- Identifiable, 3- highly appreciated
H- HISTOPATHOLOGY; C- CYTOPATHOLOGY

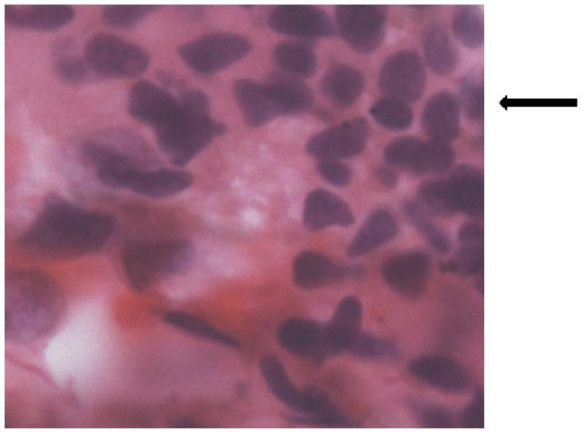
Table 4. Observer Score for Equipments Availability, Cost Effectiveness and Duration

(O) observer Criteria	O_1	O_2	O_3	O_4
Equipment availability	H-1	H-1	H-1	H-1
	C-1	C-1	C-1	C-1
Cost	H-1	H-1	H-1	H-1
	C-1	C-1	C-1	C-1
Duration	H-3	H-3	H-3	H-3
	C-1	C-1	C-1	C-1

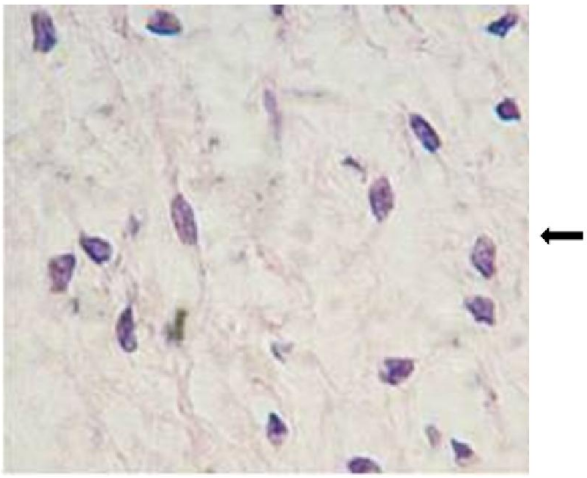
SCORE (AVAILABILITY): 1- Easily available, 2- Available at special setups
SCORE (COST): 1- Cost effective, 2- moderately expensive, 3- highly expensive
SCORE (DURATION): 1- Time effective (40-50 minutes), 2- moderately time consuming (3-4 hours), 3- more time consuming (>6 hours)
H- HISTOPATHOLOGY; C- CYTOPATHOLOGY

Cell membrane was equally identifiable in both techniques however the nuclear membrane was better appreciated in histopathological sections than in cytopathological smears. Barr-bodies were seen in both the techniques but were more highly appreciated in histopathological sections (Table 3). A densely stained mass was appreciated lying next to the nuclear membrane. The inset showed a number of cell nuclei possessing highly appreciated Barr-bodies at the periphery (Picture 1). Paucity of cellular material during cytopathology technique was observed. It also showed a few nuclei with Barr-bodies but the Barr-bodies were less appreciated in this technique (Picture 2).

The equipment was easily available for both techniques and both techniques were cost effective, though the histopathological technique was more time consuming than cytopathological technique (Table 4).



Picture 1. Pulpal tissue in histopathological section demonstrating barr-bodies (H&E STAIN; 40X)



Picture 2. Cytopathological preparation showing barr-bodies (H&E; 40X)

DISCUSSION

Human females possess two X chromosomes and hence are termed as 'HOMOZYGOUS', whereas human males are 'HETEROZYGOUS' with one X and one Y chromosome (Anoop *et al*., 2004). Mary Frances in her Lyon hypothesis (1962) stated that in cells with multiple X-chromosomes, one X-chromosome is inactivated during mammalian embryogenesis in females (Anoop *et al*., 2004). This process of inactivation of one X-chromosome is called 'LYONIZATION' and is seen in species where sex is determined by the presence of Y-chromosome or W-chromosome (Anoop *et al*., 2004). Barr-body is X-chromatin in inactive form and appears as an intra-nuclear structure. They were discovered by Murray Barr & Bertam (1949) and are identified as mass usually lying against the nuclear membrane in all female somatic cells during the Interphase of the cell cycle (Das *et al*., 2004). In human beings, the number of Barr-bodies per cell is calculated by the following formula: Barr-body/cell = Total number of X-chromosomes-1. Therefore, in females there is one Barr-body/ cell and in males, Barr-bodies are absent. Exception to this rule are observed in conditions like- Klinefelter syndrome (47, XXY in males and in females 47, XXX), Down's syndrome (trisomy 21) (Anoop *et al*., 2004). This study demonstrated that the mean Barr-body count in case of female pulpal samples was more than that observed in male samples and was more in histopathological technique than in cytopathological technique indicating that the average number of Barr-bodies seen in female somatic cells was more than that in male cells in both the techniques employed. The Barr-bodies were better appreciated in histopathologically assessed slides as there was less damage of the sample during technique procedure and the integrity of the cellular and nuclear structures was better preserved in histopathology slides as compared to cytopathology smears. In this study an extremely significant difference (P=0) was found between both the techniques. The histopathological technique was much effective in identification of Barr-bodies. In cytopathological technique, paucity of cellular material made it relatively difficult to identify the Barr-bodies. The studies by Whittaker (1975), Das (2004) and Nayar (2014) regarding Barr-bodies identification in human dental pulp tissue have reported excellent results through cytopathological technique though there is no comparison between the histopathological and cytopathological technique as per our study. Although some reported characterization of Barr-bodies and their appearance in cytological smears such as the appearance of Barr body in the cell nuclei, its location, the integrity of cell and nuclear membrane, were found to be similar with the results of the current study.

Conclusion

The mean Barr-body count was more in females than in male samples and in histopathological analysis as compared to cytopathological technique. Also cell morphology was better appreciated in histopathology than in cytopathology. Though the cytopathology technique is effective while looking for these structures in commonly studied buccal mucosal smears, the histopathology technique is more time consuming but it gives an excellent result in Barr-Body identification in dental pulp tissue.

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